MicroRNA Analysis of Archival FFPE Samples by Microarray

Application Note

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Abstract

MicroRNAs (miRNAs) have diagnostic and prognostic potential for various diseases, most notably for cancer. Microarray-based hybridization has proven to be a powerful technique for miRNA profiling. While many studies have focused on fresh-frozen (FF) tissues, other types of samples such as formalin-fixed paraffin-embedded (FFPE) samples are being explored for retrospective analysis. FFPE samples are of significant value because they are frequently the only sources of tissue available from large patient cohorts with comprehensive clinical data and long-term follow-up—spanning decades in some cases.

The primary challenge of profiling miRNAs from FFPE samples is the extraction of total RNA that appropriately retains the small RNAs. Most methods for extracting RNA from FFPE samples have been optimized for recovery of significantly longer RNAs. To enable researchers to utilize these valuable samples, we have tested various extraction methods and have identified the methods that work best in combination with the miRNA microarray profiling system using the miRNA Complete Labeling and Hyb Kit.

The extraction methods were tested using a colon cancer matched quad set (FF and FFPE of matched Normal and Cancer samples) and three lung cancer matched quad sets that had been stored for 1 to 10 years. The extracted total RNA was quantified, and 100 ng was labeled and hybridized using the Agilent miRNA Complete Labeling and Hyb Kit in conjunction with the Agilent Human miRNA microarray (V2). The data, which were analyzed with GeneSpring GX 10.0 software, demonstrated good assay reproducibility between technical replicates.

Most of the miRNAs detected in the FF-derived samples were also detected in the FFPE-derived samples. Hierarchical clustering revealed that samples stored for a shorter period of time clustered according to disease state (i.e., tumor or normal), while samples stored for longer periods clustered according to storage condition (i.e., FF or FFPE). The qRT-PCR data for selected miRNAs demonstrated high concordance with the miRNA microarray data.

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Introduction

MicroRNAs (miRNAs) are endogenous non-coding RNAs that are ~22nt in length at maturity. Mature miRNAs are known to regulate gene expression by either translational repression or mRNA degradation. This interaction has been demonstrated to occur at various stages of multiple cellular processes and has been implicated in numerous human diseases, most notably in cancer. In cancer, the altered regulation of miRNA profiles suggest a potential that these genes function as tumor suppressors and ongogenes (Slaby et al., 2007). Many studies have been conducted comparing the miRNA profiles of normal tissue versus cancerous tissue and have found cancer-specific differentially expressed miRNAs.

Specific miRNA signatures include: the under-expression of miR-143 and miR-145 and the over-expression of miR-31 and miR-21 in human colorectal cancer (CRC) (Slaby et al., 2007); over-expression of miR-424 and miR-203 in human kidney cancer; and over-expression of miR-21 and miR-205 in non-small cell lung cancer (NSCLC) (Markou et al., 2008). These cancer-specific signatures allow further classification of cancers, making miRNAs important to cancer research and potential diagnostics. The studies cited above used microarrays to profile the miRNAs in samples of high-quality total RNA extracted from fresh-frozen (FF) tissues that were handled appropriately to prevent degradation of the RNA.

FF tissues, although ideal for miRNA profiling, are not as readily available as formalin-fixed paraffin-embedded (FFPE) samples. There are more than 400 million FFPE samples that have been collected and stored for more than 10 years, providing a large source of archival tissue samples available for retrospective prognostic studies of human cancer. Extracting good quality total RNA from FFPE samples is difficult due to the cross-linkage between nucleic acids and proteins, covalent RNA modification, and dimerization of adenine groups (Masuda N. et al., 1999), as well as degradation during the fixation process and storage period. Additionally, many of the available methods for extracting total RNA have been optimized to extract longer RNAs. This excludes the smaller RNAs, including the miRNA fraction. These difficulties are further exacerbated by the lack of standards for tissue fixation and other procedures employed in the preparation of FFPE samples. Once total RNA (containing miRNA) has been obtained from FFPE samples, reliable miRNA profiles can be obtained with the Agilent miRNA microarray system using the same protocol as for fresh-frozen material.

For this study, we used quad sets: matched sample sets consisting of normal and cancerous samples in both the fresh-frozen and FFPE forms, from two different tissue types as well as several different sources. These matched quad sets enabled comparisons of differentially expressed miRNAs between the two types of tissue storage methods. The sets ranged in age from 1 to 10 years. We also tested several different total RNA extraction methods. Total RNA was extracted from the fresh-frozen samples using the miRNeasy Kit (Qiagen) and the mirVana miRNA Isolation Kit (Life Technologies). For the FFPE samples, we used the miRNeasy FFPE Kit (Qiagen) and the RecoverAll Total Nucleic Acid Isolation Kit (optimized for FFPE samples, Life Technologies) to extract total RNA. A subset of the extracted total RNA samples was tested for DNA contamination by qPCR. The extracted total RNAs were assayed for miRNA expression using the Agilent miRNA microarray system. The extraction of total RNA through the analysis using the Agilent miRNA microarray system was performed in parallel by users in different labs for a subset of the quad sets. An overview of the workflow for Agilent miRNA microarray analysis of fresh-frozen and FFPE samples is presented in **Figure 1**.

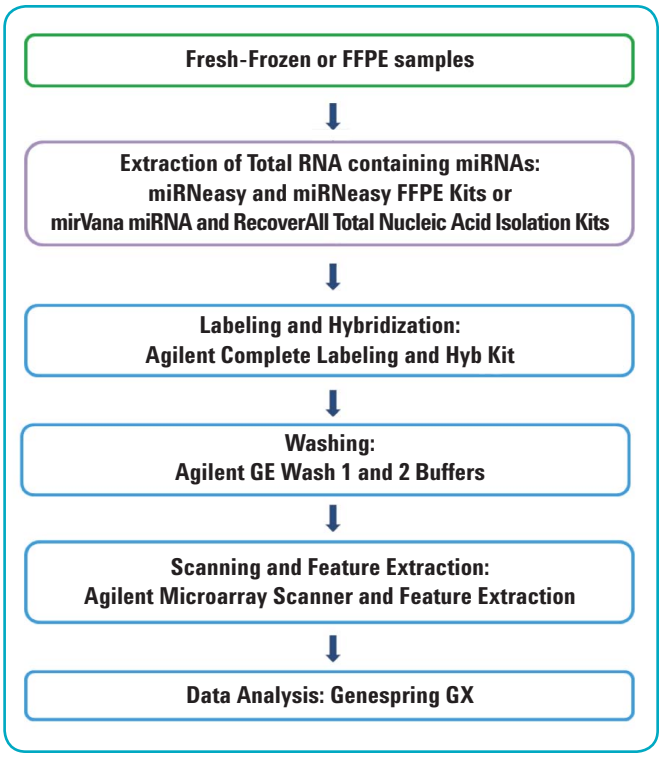

Figure 1 shows the workflow for processing fresh-frozen and FFPE samples, using the various total RNA extraction kits (purple box) in conjunction with the various steps of the Agilent miRNA microarray workflow (blue boxes).

Materials and Methods

RNA isolation and QC analysis

Quad sets: matched normal and adenocarcinoma colon tissues from both fresh-frozen (FF) and formalin-fixed paraffin-embedded (FFPE) samples, were obtained from Asterand Technologies (Detroit, MI). Additional quad sets of matched normal and non-small cell lung cancer were obtained from the UMC Groningen in the Netherlands. The quad sets ranged in age from 1 to 10 years at the time of total RNA extraction. For the fresh-frozen samples, we used the miRNeasy Kit (Qiagen) and the mirVana miRNA Isolation Kit (Life Technologies) to extract total RNA from ~25 mg of normal and tumor colon tissue, ~ 15 mg of lung normal tissue, or ~ 4.5 mg of lung tumor tissue. For the FFPE samples, total RNA was extracted from two 10-µm-thick paraffin-embedded tissue sections for colon, or from two 20-µm-thick sections for all three lung sets, using the miRNeasy FFPE Kit (Qiagen) or the RecoverAll Total Nucleic Acid Isolation Kit (Life Technologies).

Extractions were performed in either duplicate or triplicate for each quad set for each extraction method. The quality of the total RNA was assayed using the Agilent 2100 Bioanalyzer Eukaryote Total RNA Nano or Pico assay. The presence of small RNAs in the fresh-frozen samples was assayed using the Agilent Bioanalyzer Small RNA assay. These assays were also used with the RNA derived from the FFPE samples with the appreciation that these samples were potentially highly degraded—which would be confirmed by the assay results. The quantity of the extracted total RNA was determined using the Nanodrop.

Due to a number of samples having A260/280 and A260/230 ratios <1.8, all total RNA samples were further purified using a nucleic acid purification column (Bio-Rad Micro Bio Spin 6 Columns Cat # 732-6221). A buffer exchange with nuclease-free water was performed, and then 25uL to 50uL of the concentrated sample was applied to the column. The presence of DNA contamination, which would result in inaccurate RNA quantitation was determined by a SYBR Green qPCR assay. The assay was conducted using 100ng of total RNA in a Brilliant SYBR Green qPCR master mix (Stratagene P/N 600548), using the DNA-specific Quantos qPCR Normalization Primers (Set 1) found in the Stratagene SideStop Kit (Stratagene P/N 400908). Analysis was performed using the MX3000P real-time PCR system (Stratagene P/N 401403).

Total RNA labeling

For each quad set and extraction method, 100ng of total RNA was labeled using the Agilent miRNA Complete Labeling and Hyb Kit (P/N 5190-0456,) in duplicate or triplicate from each of the three extraction replicates of the four samples, for a total of 24 or 36 labeling reactions depending on the user. The samples were labeled according to the procedure outlined in the Agilent miRNA Microarray System with miRNA Complete Labeling and Hyb Kit Protocol manual (Version 2.0 P/N G4170-90011).

Hybridization and washing

Each of the labeled samples were combined with Agilent 10x Blocking Agent and Agilent 2x Hi-RPM Hybridization Solution (both components of the Agilent miRNA Complete Labeling and Hyb Kit, P/N 5190-0456). Prior to array hybridization, hybridization mixtures were denatured at 100°C for 5 minutes and then immediately snap-cooled in ice water for an additional 5 minutes. The samples were hybridized to the Agilent Human V2 miRNA Microarrays (P/N G4470B). Each slide contains eight identical microarrays containing probes for 723 human and 73 human viral miRNAs. Hybridization was carried out at 20 RPM at a temperature of 55°C for 20 hours. Following hybridization, the arrays were washed according to the procedures outlined in the Agilent miRNA Microarray System with miRNA Complete Labeling and Hyb Kit Protocol manual (Version 2.0 P/N G4170-90011).

Microarray scanning and data analysis

Scanning and image analysis were performed using the Agilent DNA Microarray Scanner (P/N G2565BA) equipped with extended dynamic range (XDR) software according to the Agilent miRNA Microarray System with miRNA Complete Labeling and Hyb Kit Protocol manual (Version 2.0 P/N G4170-90011). Feature Extraction Software (Version 10.5) was used for data extraction from raw microarray image files using the miRNA_105_Dec08 FE protocol. Data visualization and analysis was performed with GeneSpring GX (Version 10.0) software.

Results

RNA yield and purity

RNA yields and purity were assessed to ensure that the material obtained was of sufficient quality and quantity to be labeled and hybridized for miRNA profiling analysis. The RNA yield obtained from the four quad sets was evaluated using a NanoDrop

spectrophotometer. The RNA yield obtained from 2 x 10-micron thick FFPE human colon tissue sections was about 8 µg for normal tissue and 41 µg for tumor tissue. The yield from approximately 25 mg of fresh-frozen samples was about 12 µg and 32 µg for normal and tumor, respectively. The total RNA yields for the three lung quad sets were lower than for the colon, most likely due to the smaller size of the tissues within the sections and the tissue type.

Both the FFPE derived and fresh-frozen-derived total RNA samples had high purity ratios (A260/280 and A260/230), indicating the total RNA isolated with the extraction kits tested was of sufficient quantity and quality for miRNA profiling using the Agilent miRNA microarray system. **Table 1** shows the A260/280 and A260/230 ratios as well as the concentration and yields obtained for the 10-year-old lung quad set using the Qiagen kits. A qPCR assay revealed approximately one percent or less DNA contamination in the total RNA extracted for both extraction methods used.

The total RNA quality was assayed using the Agilent 2100 Bioanalyzer Eukaryote Total RNA Nano or Pico assay. The fresh-frozen samples consistently had RNA integrity numbers (RINs) greater than 7, indicating that they were of good quality. The FFPE samples consistently had RINs of approximately 2, suggesting that the samples were degraded, as expected for this type of sample. **Figure 2** shows the electropherograms for the 10-year-old lung quad set using the Qiagen extraction kits.

Storage	Disease State	Conc (ng/µl)	A260/A280	A260/A230	Yield (µg)
FFPE	Normal Lung	788.99	1.8	1.9	23.67
FFPE	Tumor Lung	969.57	1.8	1.9	29.09
FF	Normal Lung	252.29	1.9	1.8	10.09
FF	Tumor Lung	277.19	1.9	2.06	11.09

Table 1. The Qiagen miRNeasy and miRNeasy FFPE Kits for total RNA extraction results from a 10-year-old lung quad set. The A260/280 and A260/230 ratios are both ≥1.80, indicating that the RNA was isolated with very few contaminants.

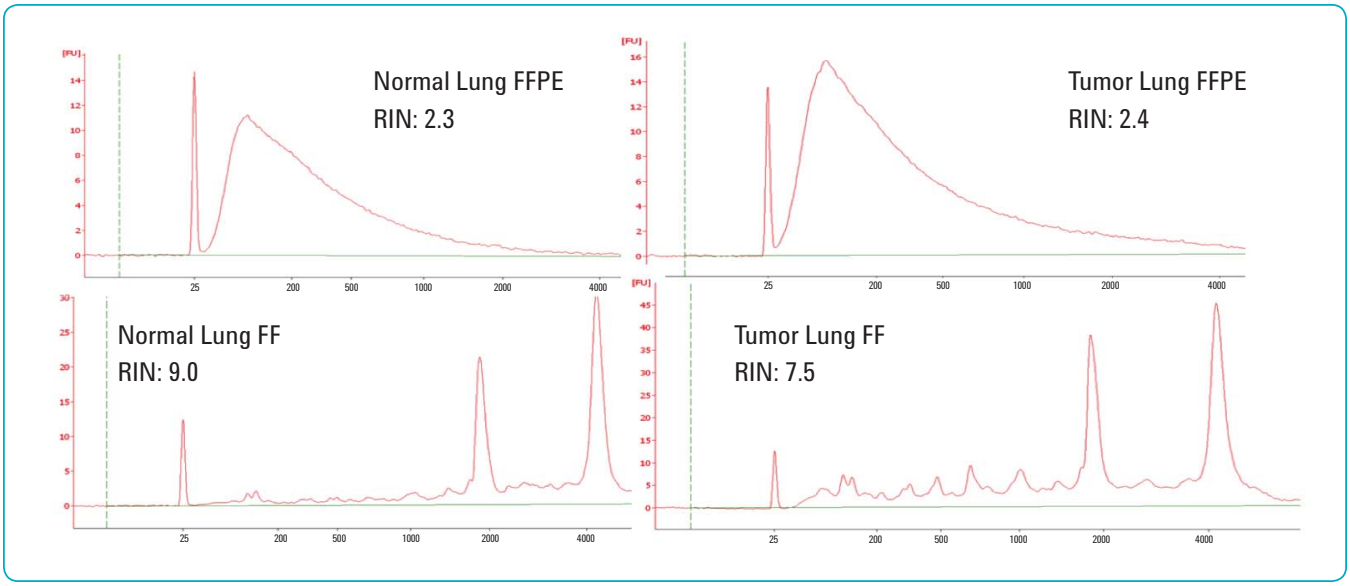

Figure 2. The RNA quality was analyzed using the Agilent 2100 Bioanalyzer – Eukaryote Total RNA Nano assay. The majority of RNA fragments isolated from the 10-year-old lung quad set for FFPE normal and FFPE tumor tissues were between 100 and 500 bp. The low RNA integrity number (RIN, around 2.0) was typical for FFPE extractions as shown in the top two traces while the higher RINs were typical for the fresh-frozen extraction as shown in the bottom two traces.

The presence of miRNA in the total RNA was assayed using the Agilent 2100 Bioanalyzer Small RNA assay. The fresh-frozen samples had miRNA percentages mostly in the range of 5 to 20 with distinct peaks detected. The FFPE samples consistently had miRNA percentages reported at greater than 20 without distinct peaks, a result which was expected due to the degraded nature of the samples. Similar results were obtained for both extraction kits used to extract total RNA from the fresh-frozen and FFPE samples. **Figure 3** below shows the small RNA electropherograms for the 10-year-old lung quad set using the Qiagen extraction kits.

Gene list concordance between FFPE and FF samples

As a measure of how successful the extraction of total RNA to include miRNA from FFPE samples was, we compared the number of detected miRNAs between the FF and the FFPE samples for both disease states as shown in **Figure 4**. MiRNAs are determined to be detected during data extraction using Feature Extraction software; with the result output as a gIsGeneDetected flag, which was loaded into GeneSpring GX 10.0. In general, slightly more miRNAs were detected in the FF samples than the FFPE samples, but for some quad sets the opposite was true.

After determining that the technical replicates had high concordance, we wanted to further understand the correlation of detected miRNAs. We plotted the extraction and labeling replicate average 'gTotalGeneSignal' for each storage

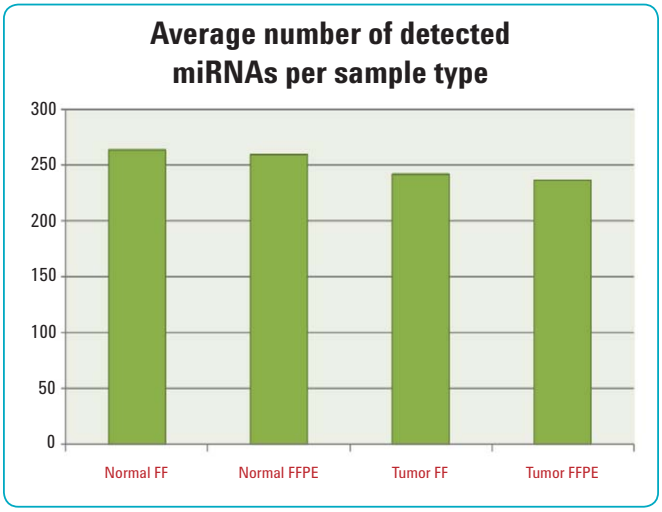

Figure 4. The average number of detected miRNAs per sample type for the colon quad set. The number of detected miRNAs is slightly lower for the FFPE samples than the FF colon samples, indicating that retention of the miRNAs during storage and total RNA extraction was achieved.

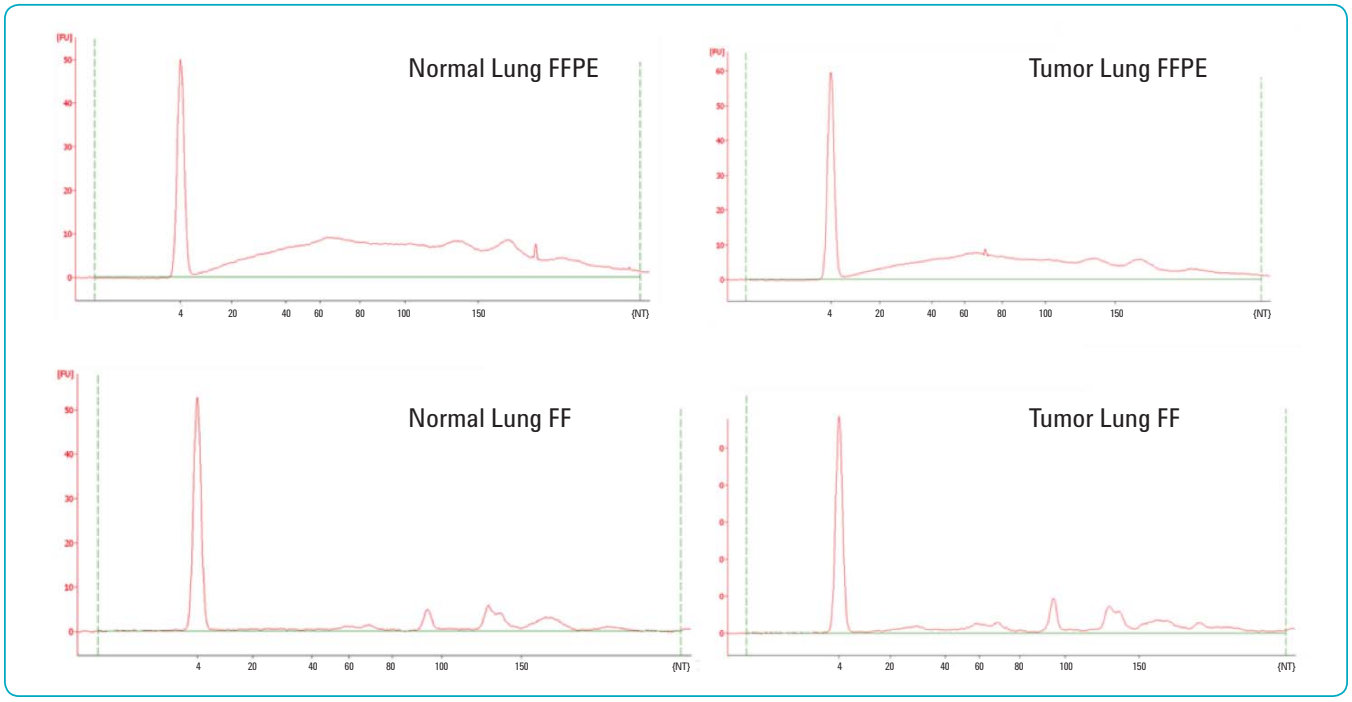

Figure 3. The presence of miRNAs or small RNAs was assayed using the Agilent 2100 Bioanalyzer – Small RNA assay. The electropherogram for the fresh-frozen 10-year-old lung samples had distinct peaks of the small RNAs as shown in the bottom traces. The electropherogram for the FFPE 10-year-old lung samples had broad bands without distinct peaks, indicative of degraded samples as shown in the top traces.

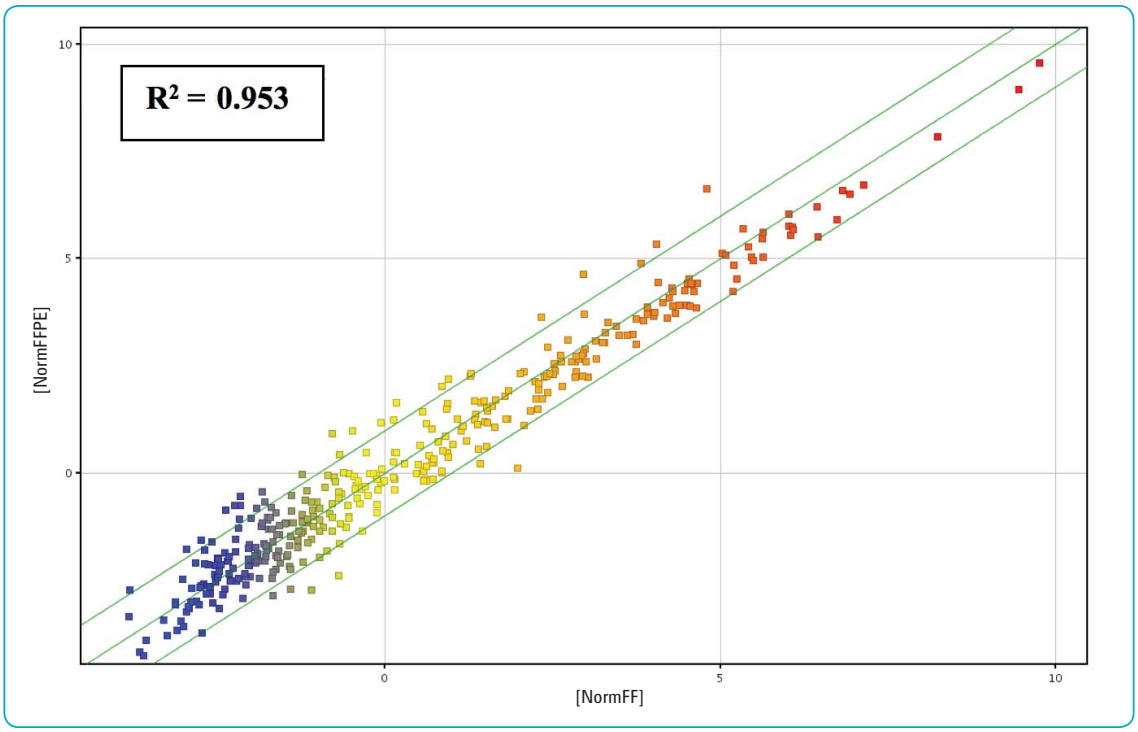

Figure 5a. Correlation of miRNA profiles between FF and FFPE normal colon samples.

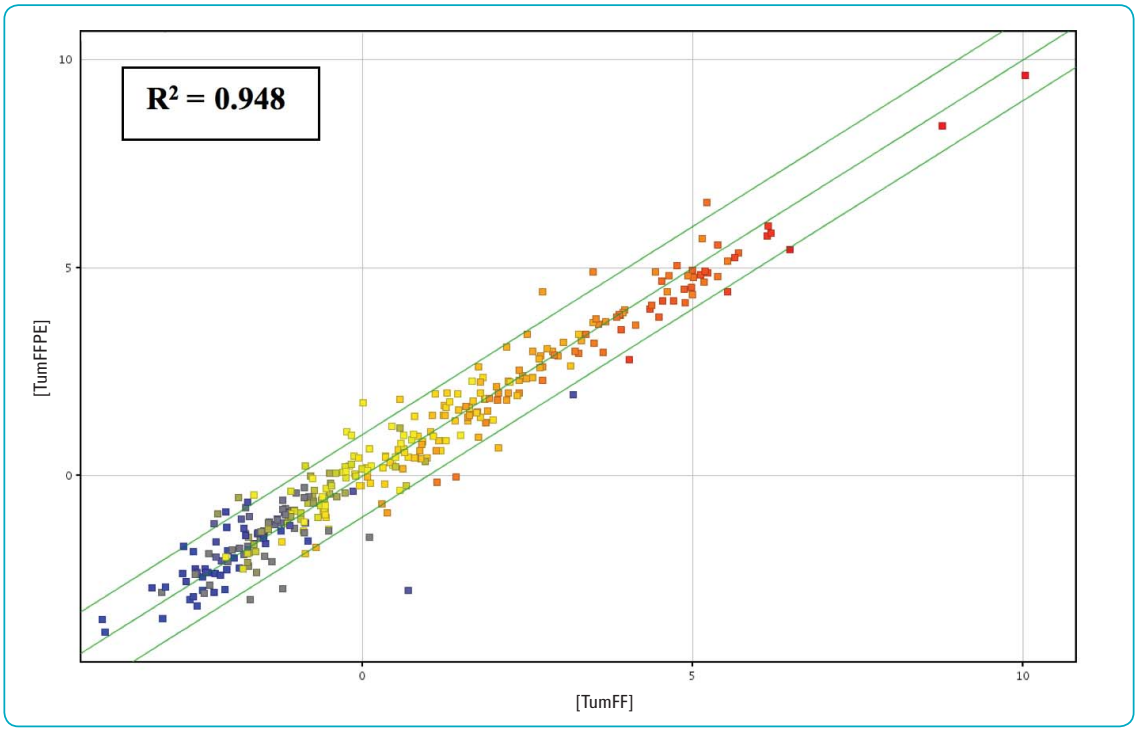

Figure 5b. Correlation of miRNA profiles between FF and FFPE tumor colon samples.

Figure 5. Correlations of human miRNA profiles between FF and FFPE colon samples. The average normalized TotalGeneSignal of detected miRNAs for all the replicates of a given tissue state and sample type demonstrate strong correlation between FF (X-axis) and FFPE (Y-axis) sample types. Figure 5a shows the correlation between the normal samples and 5b shows the correlation between the tumor samples.

condition within a disease state. **Figure 5** illustrates the tight correlation of the human miRNA profiles based on disease state for the colon quad set.

Hierarchical Clustering of the Quad Sets

To understand the impact of the storage condition on the miRNA expression profiles, hierarchical clustering was performed in GeneSpring GX10 using Euclidean distance metrics and centroid linkage rule of the average replicates per condition. The hierarchical clustering for all the quad sets, regardless of source, tissue type or user, revealed that the miRNA profiles clustered primarily based on disease state rather than storage condition (**Figure 6**), with the exception of the 10-year-old lung sample, which clustered primarily on storage condition (**Figure 7**).

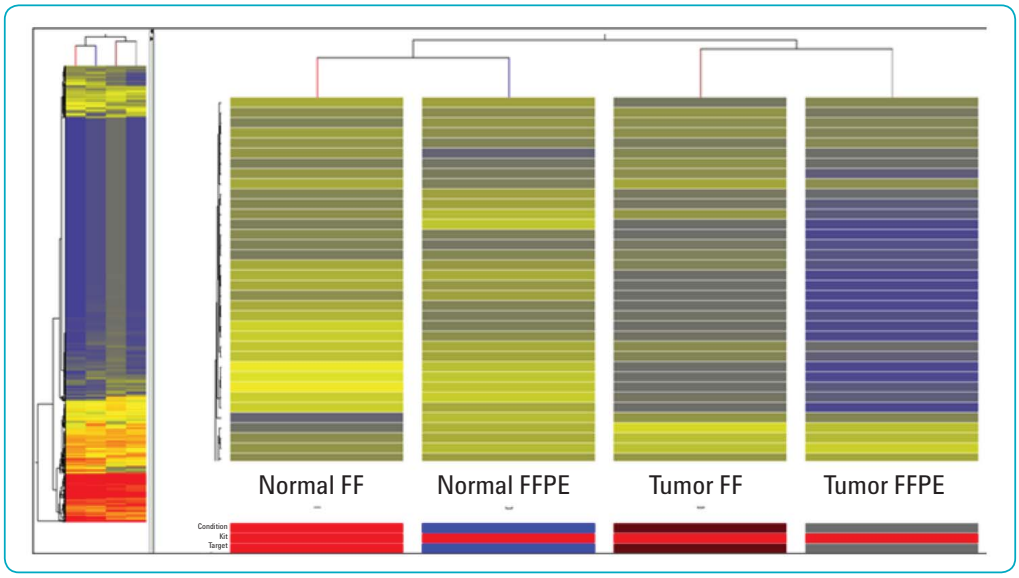

Figure 6. Hierarchical clustering reveals that miRNA profiles cluster primarily based on disease state, with normal samples clustering separately from tumor samples. Clustering in GeneSpring GX10 used Euclidean distance metrics and centroid linkage rule of the average replicates for a two-year-old lung quad set.

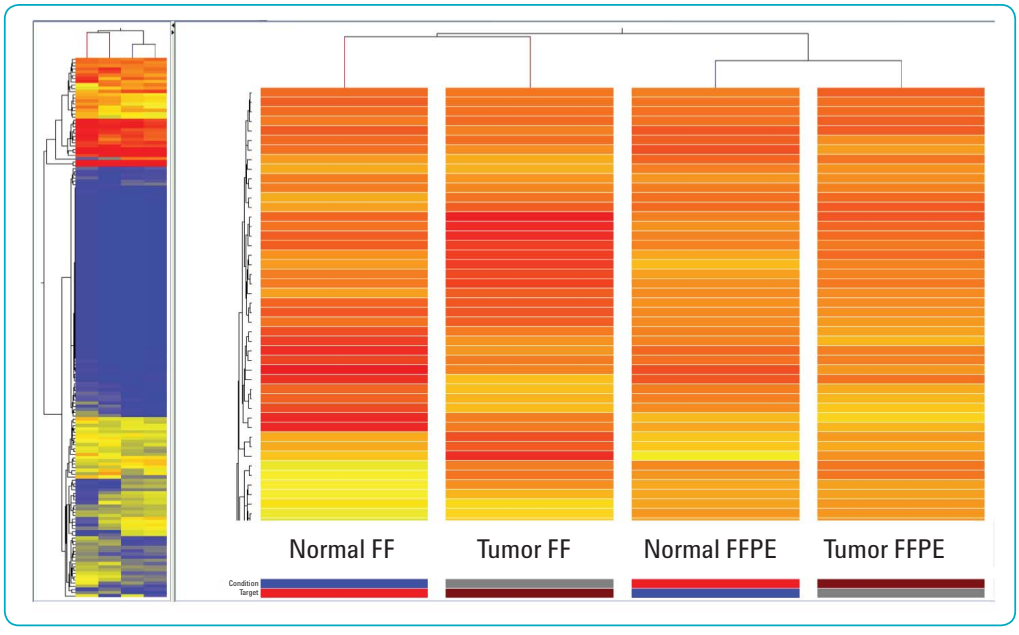

Figure 7. Hierarchical clustering reveals that miRNA profiles cluster primarily based on storage conditions, with FF samples clustering separately from FFPE samples. Clustering in GeneSpring GX10 used Euclidean distance metrics and centroid linkage rule of the average replicates for a 10-year-old lung quad set.

Statistically significant miRNA expression differences between normal and tumor samples

To understand the value of FFPE samples, it is important to know if the miRNA signatures are retained compared to fresh-frozen tissue. We observed that miRNAs found to be significantly differentially expressed between tumor and normal samples in FF samples were also found to be differentially expressed in FFPE samples. The volcano plots (**Figure 8**) show the miRNAs with significant differential expression (in red) between the normal and tumor samples for each storage condition. In this figure, the magnitude of fold change between the normal and tumor conditions is compared to the statistical significance (corrected p-value of <0.05) of the fold change (**Figure 8**). Comparison of the differentially expressed miRNAs across the different storage conditions reveals that more than 70 percent of the miRNAs differentially expressed between the normal and tumor fresh-frozen samples were also found to be differentially expressed in the FFPE samples. MiR-143, -145 and -31 are consistently differentially expressed both in the FF and FFPE sample types; consistent with previously published data (Slaby et al., 2007).

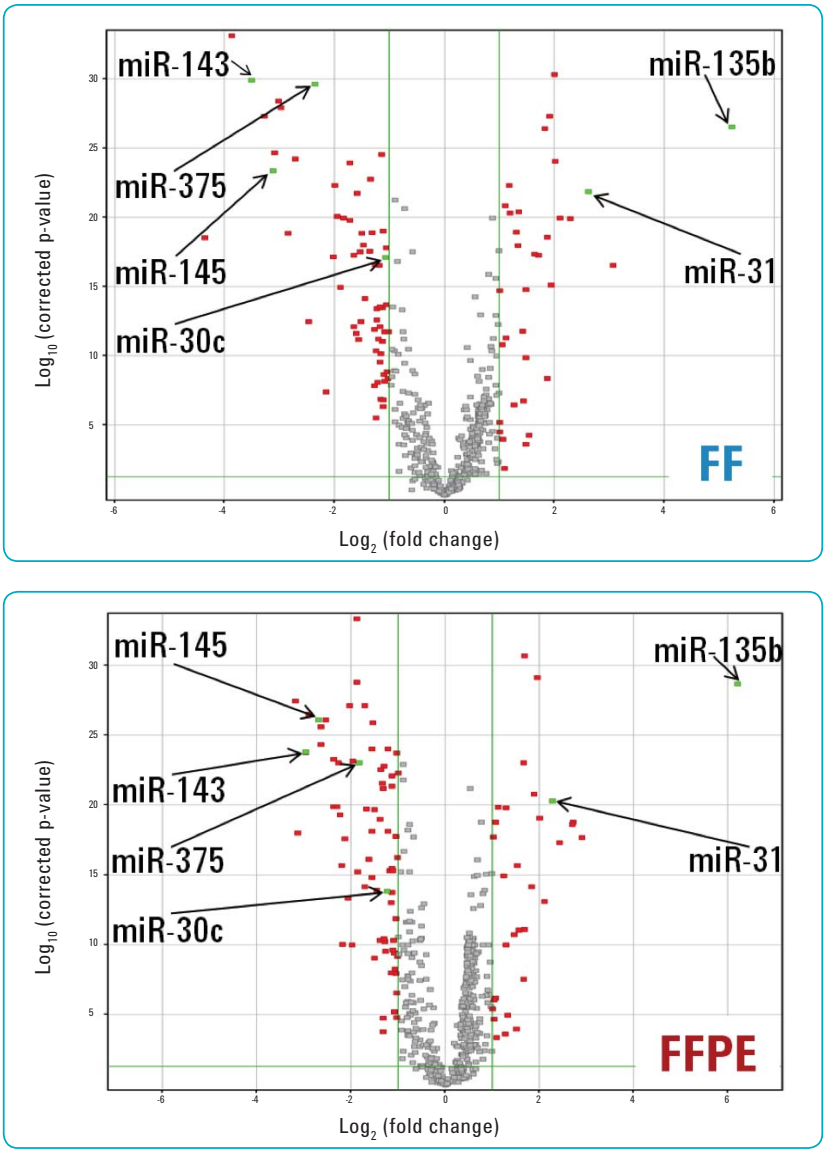

Figure 8. Analysis of the differential expression of the FF and FFPE storage conditions demonstrates hundreds of differentially expressed miRNAs in both conditions for the colon samples. The $\log_2$ fold change values are plotted on the x-axis of the volcano plots and are compared to the negative $\log_{10}$ corrected p-values on the y-axis. MiRNAs with an absolute differential expression fold change of at least two-fold with a corrected p-value of at least 0.05 are colored red. The green lines on the plots indicate the significance cut-offs of two-fold differential expression at a corrected p-value of 0.05.

Verification of specific miRNA expression through qRT-PCR

To verify the miRNA data, we selected a few miRNAs and tested their expression levels using qRT-PCR (**Figure 9**). Those miRNAs that showed differential expression in specific tumors, including miR-143 and miR-145 (underexpressed in colorectal cancer), and miR-31 and miR-21 (overexpressed in colorectal cancer), were selected for qRT-PCR analysis along with some non-differentially expressed miRs. qRT-PCR data demonstrate strong correlation with the miRNA microarray data for the nine miRNAs tested using the colon samples as shown in **Figure 9a**. Eight of those miRNAs (all but miR-31) were also tested with the lung quad sets as illustrated in **Figures 9b** and **9c**. The FF and FFPE data demonstrated good correlation; miRNAs up-regulated in tumor as compared to normal in FF were also up-regulated in the FFPE samples. The same was true for down-regulated and non-differentially expressed miRNAs.

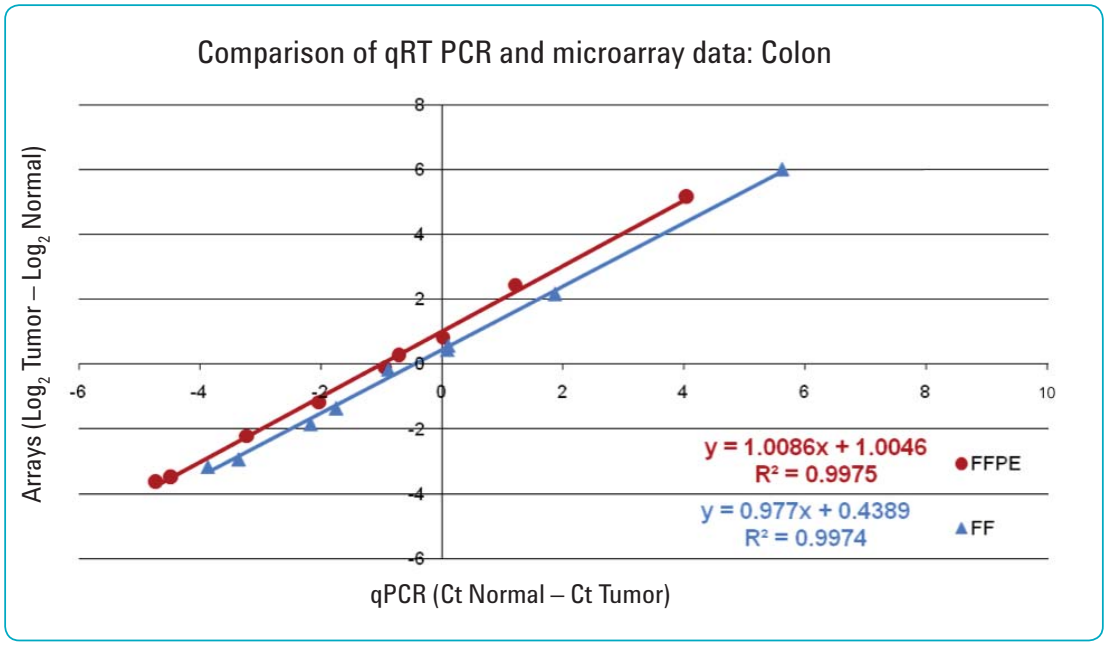

Figure 9a. Comparison of qRT-PCR and microarray data for colon quad set.

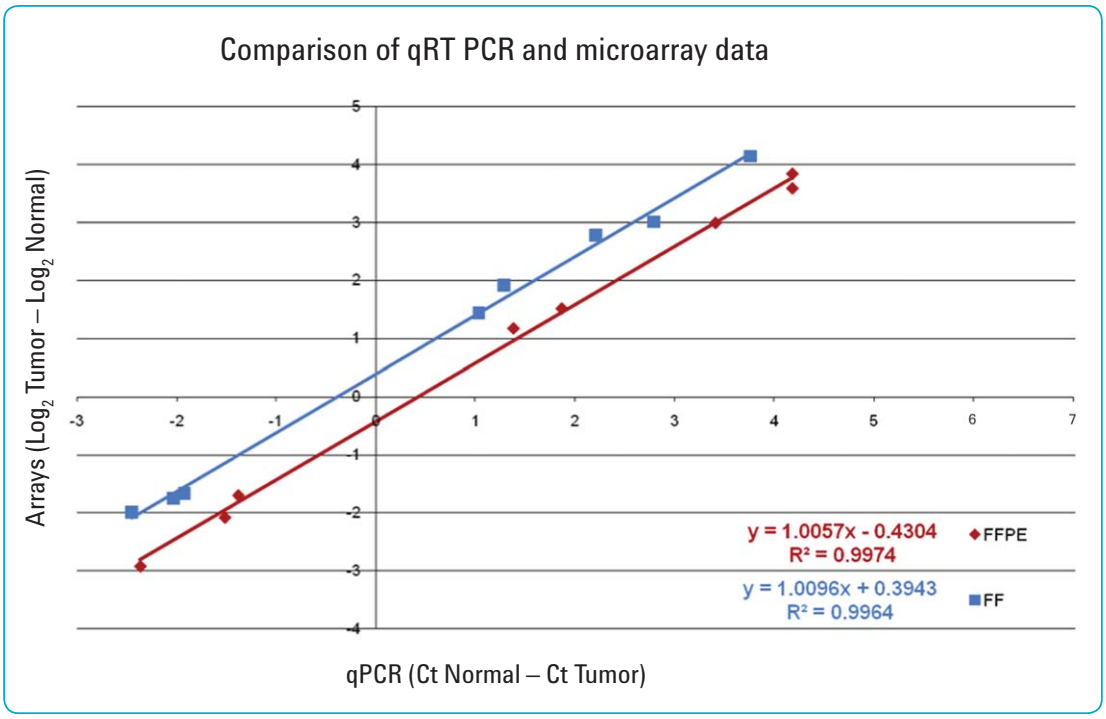

Figure 9b. Comparison of qRT-PCR and microarray data for 1-year-old lung quad set.

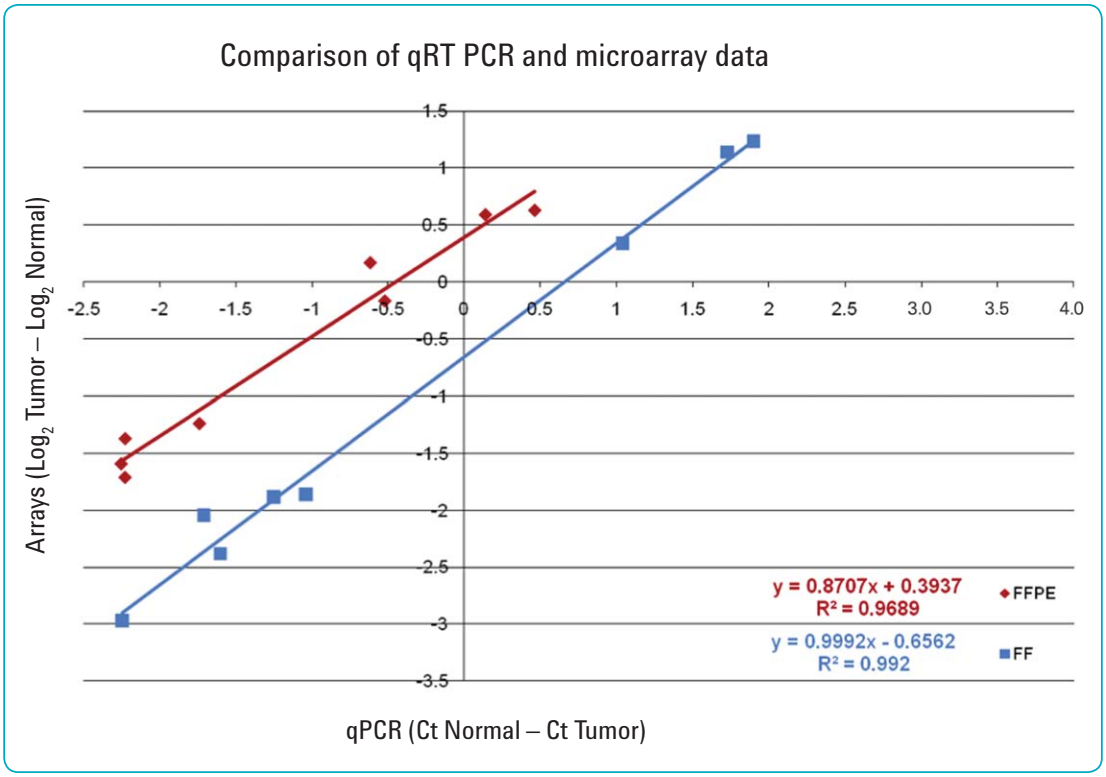

Figure 9c. Comparison of qRT-PCR and microarray data for 10-year-old lung quad set.

Figure 9. The scatter plots demonstrate high correlations for qRT-PCR to microarray data for various quad set samples. The differential expression values between the normal and tumor samples of the FF and FFPE storage conditions are also highly concordant. These data verify that using the Agilent miRNA microarray system generates reliable data for profiling miRNAs in FFPE samples.

Conclusions

Formalin-fixed paraffin-embedded (FFPE) archival tissue samples are a valuable source of material for retrospective prognostic miRNA profiling studies of human cancer. These samples can be used in miRNA profiling studies with the Agilent miRNA profiling system, using either of the RNA extraction methods described. The data shown here demonstrate that FFPE samples can produce reliable miRNA profiles using the workflow shown in **Figure 1**. Generally the differential expression profiles obtained for FFPE samples correlate well to those of matched fresh-frozen samples—regardless of source, user and tissue type. Retrospective studies using FFPE samples can be extremely valuable because of the vast amount of clinical parameters and outcomes associated with these types of samples. The results of these studies can have great impact on prognosis and diagnosis going forward.

References

1. Slaby et al. (2007) **Altered Expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer.** *Oncology 2007;72:2397-402*

2. Markou et al. (2008) **Prognostic value of mature microRNA-21 and microRN-205 overexpression in non-small cell lung cancer by quantitative real-time RT-PCR.** *Clinical Chemistry 2008;54:1696-1704*

3. Masuda N et al. (1999) **Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples.** *Nucleic Acids Research (27) 22:4436-4443*

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